Our laboratory focuses on two general areas: signal transduction and growth control. Much of our work centers about the RAS proteins, small guanine-nucleotide-binding proteins, which reside on the inner surface of the plasma membrane, that have been highly conserved in evolution and play a critical role in mediating signals that control cellular growth and other cellular events. We study the RAS proteins in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and in mammalian cells. In addition to RAS, we also study the families of mammalian cAMP phosphodiesterases, which play important roles in modulating the response of cells to cAMP. We plan to continue our studies of ROS, an oncogene that is expressed specifically in glioblastomas. Finally, we are developing a method for genomic difference cloning, which should enable scientists to discover new genetic abnormalities in cancer cells and search more effectively for new pathogenic organisms.

### RAS in *Saccharomyces cerevisiae*


There are two RAS proteins in *S. cerevisiae*, encoded by the *RAS1* and *RAS2* genes. They are highly ho-
mologous to the mammalian RAS genes. These yeast proteins are essential for growth and control the activity of adenyl cyclase, the product of the CYRI gene. Activation of RAS2 by point mutation (e.g., \textit{RAS2}^{val19}) leads to activation of the cAMP signaling pathway and a consequent cluster of cellular phenotypes, including loss of tolerance to heat shock and sensitivity to nitrogen starvation. We have previously described the cloning of many genes that function along the RAS pathway, including \textit{CDC25}, which encodes a factor that probably functions to regulate RAS by controlling guanine nucleotide exchange; \textit{CYRI}, \textit{BCY1}, which encodes the regulatory subunit of the cAMP-dependent protein kinase (cAPK); \textit{TPK1}, \textit{TPK2}, and \textit{TPK3}, which each encode catalytic components of the cAPK; and \textit{PDE1} and \textit{PDE2}, which encode the low- and high-affinity cAMP phosphodiesterases, respectively. Using these genes, we have demonstrated a powerful feedback inhibition that maintains this system in homeostasis. We have demonstrated that the mammalian RAS proteins have been sufficiently conserved in evolution that they can function in yeast and stimulate yeast adenyl cyclase in vitro. We have further demonstrated that RAS proteins must bind guanosine triphosphate in order to stimulate adenyl cyclase. Finally, we have demonstrated through genetic analysis that it is highly likely that RAS proteins have additional functions in yeast (Wigler et al., \textit{Cold Spring Harbor Symp. Quant. Biol.} 53: 649 [1988]).

Many of the most essential questions about RAS in yeast remain unsettled: How is RAS itself controlled? How does RAS control adenyl cyclase? Are there intermediate proteins required for this function? What are the other functions of RAS in yeast? We have made progress on these questions.

\textbf{RAS in \textit{S. cerevisiae}: How Is It controlled?}

R. Ballester, E. Chang, K. Ferguson, C. Nicolette, A. Vojtek

We have previously shown that RAS can stimulate its yeast effector only when bound to GTP. Thus, RAS may be controlled by factors that influence nucleotide binding. Two genes have been discovered in \textit{S. cerevisiae} that are likely to encode proteins involved in this type of control of RAS. The first is \textit{CDC25}, which encodes a product that probably catalyzes guanine nucleotide exchange on RAS. The second is \textit{IRA1}, which encodes a protein that probably catalyzes GTP hydrolysis by RAS (Tanaka et al., \textit{Mol. Cell. Biol.} 9: 757 [1989]). \textit{IRA1} has slight homology with a mammalian gene called \textit{GAP} (Trahey et al., \textit{Science} 242: 1697 [1988]). \textit{GAP} has been shown to induce GTP hydrolysis by RAS (Trahey and McCormick, \textit{Science} 238: 542 [1987]). It has been proposed by other investigators that \textit{GAP} is the effector for RAS (Adari et al., \textit{Science} 240: 518 [1988]). In contrast, genetic analysis strongly suggests that \textit{IRA1} protein is involved with the down regulation of RAS (Tanaka et al., \textit{Mol. Cell. Biol.} 9: 757 [1989]), and more specifically with its feedback control (Wigler et al., \textit{Cold Spring Harbor Symp. Quant. Biol.} 53: 649 [1988]; Ballester et al., \textit{Cell} 59: 681 [1989]; Tanaka et al., \textit{Mol. Cell. Biol.} 9: 757 [1989]). We have found that mammalian GAP, when expressed in yeast, does indeed down-regulate RAS (Ballester et al., \textit{Cell} 59: 681 [1989]). It down-regulates both wild-type RAS2 and mammalian Ha-ras, when expressed in yeast, but not the activated RAS2\textit{val19} protein, and can genetically complement \textit{ira1} yeast. These results suggest to us that mammalian GAP may be involved in the feedback inhibition of RAS in mammalian cells.

cAMP levels in yeast are regulated by glucose (Thevelein and Beullens, \textit{J. Gen. Microbiol.} 131: 319 [1985]). Other nutrient effects may be mediated through the cAMP effector pathway. We do not know the chain of events by which these signaling events occur. Other gene products besides those of \textit{CDC25} and \textit{IRA1} may be involved. To explore this area, we have begun searching for genes that, when overexpressed in yeast, can suppress the loss of \textit{IRA1} or can suppress the loss of \textit{CDC25}. Several new candidate yeast genes have been identified, and they will be characterized by sequence, genetic, and biochemical analyses. To perform biochemical analysis, we must first find conditions under which some component can affect the nucleotide bound to RAS. We are in the process of purifying CDC25 protein from yeast to determine under what conditions it can catalyze nucleotide exchange. We have shown that dominant interfering mutants of RAS can block function of wild-type RAS (Powers et al., \textit{Mol. Cell. Biol.} 9: 390 [1989]). Interference is suppressed by overexpression of CDC25. We therefore proposed that these interfering RAS mutants form stable complexes with CDC25. We are in the process of testing this hypothesis by biochemical studies.

Our focus on the control of RAS is CDC25. For
reasons stated below, we believe it is likely that 
CDC25 has a mammalian homolog. Our studies in 
yeast thus will help guide experiments in mammalian 
cells. The fundamental question, both in yeast and 
in mammals, is the nature of the ultimate initiating 
signal for the pathway. In yeast, this initiating sig-
 nal may originate from within the cell, perhaps a con-
 sequence of the metabolic state of the cell. There may 
be a similar initiating signal in mammalian cells.

RAS in S. cerevisiae: Its 
Interactions with Adenylyl 
Cyclase

R. Ballester, J. Colicelli, K. Ferguson, J. Field, J. Gerst, 
T. Michaeli, M. Riggs, L. Rodgers, A. Vojtek

We showed several years ago that the major effects 
of RAS proteins in yeast were explainable by their 
stimulation of adenylyl cyclase and that RAS pro-
teins do indeed stimulate adenylyl cyclase in vitro. 
It has remained an open question whether this in-
teraction is direct or whether it requires the presence 
of intermediary proteins. To help resolve this ques-
tion, we are using extensive genetic and biochemical 
approaches. S. cerevisiae adenylyl cyclase made in 
Escherichia coli is neither full length nor RAS-
responsive (unpublished results). To purify the 
adenylyl cyclase complex from yeast, we developed 
a method of epitope fusion and utilized immunoaf-
finity chromatography. This epitope fusion method 
is widely applicable for studying stable protein com-
plexes. The adenylyl cyclase purified by this method 
copurifies with a tightly associated subunit with an 
apparent molecular weight of 70K (Field et al., Mol. 
Cell. Biol. 8: 2159 [1988]). We call this protein CAP 
for cyclase-associated protein. We have raised poly-
clonal antisera to CAP and identified the cDNA en-
coding CAP (Field et al., Cell [1990] in press) by 
screening an S. cerevisiae cDNA expression library 
(a generous gift from J. Kuret, Structure Section) 
with this antisera.

We have found that CAP is identical to a gene 
we had previously isolated, called SUPC. supC was 
a mutation that suppressed the phenotypes of activ-
ated RAS. In fact, cells that have disruptions of 
CAP do not respond to activated RAS2wa1ly, and the 
adenylyl cyclase purified from such yeast does not 
respond to RAS proteins in vitro.

We are currently testing if the coexpression of 
CAP and adenylyl cyclase in E. coli is sufficient to 
generate a RAS-responsive complex. At present, we 
know that these two proteins do form a complex 
when coexpressed in E. coli, but we do not yet know 
if other factors are required to form a RAS respon-
sive complex. We are continuing the search for such 
factors by both biochemical and genetic means.

CAP is itself an interesting protein. Preliminary 
data suggest that it is conserved in evolution (see sec-
tion on S. pombe, below). Disruptions of CAP lead 
to a severe phenotype that, at present, can best be 
explained by a failure to manage amino acid metab-
olism. We are in the process of testing this hypothe-
sis and are looking at the interaction of CAP with 
GCN4, a protein involved in general amino acid me-
tabolism (Hinnebusch, Mol. Cell. Biol. 5: 9 [1985]). 
The primary structure of CAP indicates that it con-
tains two domains, separable by a stretch of prolines. 
Work in progress suggests that each domain has a 
distinct function: The amino-terminal domain is re-
quired for RAS responsiveness and the carboxy-
terminal domain is required for amino acid response. 
We will continue to explore the function of CAP in 
an attempt to understand its role in mediating RAS 
effects, its role in mediating other signal transduc-
tion pathways in yeast, and its role in the evolution 
of signal transduction pathways.

We have also investigated the domains of adenylyl 
cyclase that appear to be required for interactions 
with RAS by assaying the RAS responsiveness of mu-
tant adenylyl cyclase molecules in vitro. Figuring 
prominently in this work is a domain of cyclase that 
we call the leucine-rich repeat. The leucine-rich re-
peat occurs in the middle third of the adenylyl cy-
clase molecule and is composed of about 25 units 
of a consensus motif 23 amino acids in length. This 
free is punctuated by proline and asparagine and 
occupies a domain of cyclase that 
contains leucine or an aliphatic amino acid every two 
to three residues. Similarly organized repeats have 
now been noted in a number of mammalian and in-
sect proteins that form stable complexes with other 
proteins (Field et al., Science 247: 464 [1990]). Our 
work has shown that an adenylyl cyclase molecule 
with an amino-terminal deletion within 100 amino 
acids of the leucine-rich repeat is still fully RAS-
press). Deletions within the remaining molecule de-
stroy RAS responsiveness. Small inframe insertion 
mutations reveal a different picture. Such mutations 
are generally without effect, except within the 
leucine-rich repeat. These data indicate that large-
scale spacing of the adenylyl cyclase molecule is crit-
ical to its ability to respond to RAS, and the struc-
tural requirements within the leucine-rich repeat are especially rigorous.

We have discovered a second approach to studying RAS/target interactions. Mutant forms of adenylyl cyclase can actually interfere with the function of RAS, as evidenced by their ability to restore heat-shock resistance to strains carrying the RAS2val19 mutation (Field et al., Science 247: 464 [1989]). This observation led us to map the minimum region of the adenylyl cyclase molecule capable of interfering with RAS to the leucine-rich repeat itself. These studies also led to the design of genetic screens for the mammalian effectors of RAS (see below).

RAS in S. cerevisiae: Its Other Functions
T. Michaeli, A. Vojtek, H.-P. Xu

Although most of the functions of RAS in S. cerevisiae can be explained by effects on the cAMP signaling pathway, not all of its functions can be so readily explained (Wigler et al., Cold Spring Harbor Symp. Quant. Biol. 53: 649 [1988]). First, ras1-ras2- spores are not viable, whereas cyr1- spores are. Second, cells that are ras1- ras2- and suppressed by high-copy kinase genes, such as TPK1 and SCH9, are temperature sensitive, whereas cells that are cyr1- and suppressed by these kinase genes are not temperature sensitive.

There is a third effect of RAS that is not readily explained by its effects on adenylyl cyclase. We have found that high levels of expression of an Ha-ras protein that has lost its carboxy-terminal processing site (e.g., Ha-ras186) can suppress the phenotype of the RAS2val19 gene in yeast (Michaeli et al., EMBO J. 8: 3039 [1989]). Our biochemical and genetic analyses suggest that these types of Ha-ras mutants do not interfere with the processing of RAS2val19 or its interaction with adenylyl cyclase. Moreover, the mutant Ha-ras proteins must be bound with GTP to interfere. Our results are most readily explained if the mutants of Ha-ras bind to a second RAS effector, effectively competing for its binding with membrane-bound RAS. We think that this function has been conserved in evolution, since similar interfering effects of defective Ha-ras proteins are seen in Xenopus oocytes (Gibbs et al., Proc. Natl. Acad. Sci. 86: 6630 [1989]). Several candidate yeast suppressor genes of these mutant Ha-ras genes have been found and are being analyzed. These studies may lead to the discovery of RAS effector pathways that are truly conserved in eukaryotes.

RAS in S. pombe
M. Kawamukai, M. Riggs, L. Rodgers, Y. Wang, H.-P. Xu, D. Young

We have initiated the study of RAS in S. pombe, a fission yeast that is very diverged from S. cerevisiae. At the nucleotide level, S. pombe appears as diverged from S. cerevisiae as it is from mammals. In this organism, there is a single known homolog of RAS, called ras1 (Fukui and Kaziro, EMBO J. 4: 687 [1985]). ras1- cells are viable, round, sporulation-defective, and mating-deficient (Fukui et al., Cell 44: 329 [1986]). ras1 does not appear to operate through adenylyl cyclase. These differences between S. pombe and S. cerevisiae make the study of ras1 in S. pombe particularly attractive.

We have focused on the function of ras1 and have utilized two approaches. First, we are in the process of analyzing the adenylyl cyclase system of S. pombe. Knowledge of this system will allow us to compare and contrast S. pombe and S. cerevisiae RAS-responsive systems. We will be able to test S. cerevisiae components in S. pombe to learn more about their function. In particular, we will be able to learn if these components are RAS-specific or adenylyl-cyclase-specific. Second, we are in the process of a genetic analysis of suppressors of ras1-deficient cells, in the expectation that some suppressors will encode proteins in the ras1 effector pathway. Progress has been made in both of these approaches. We have identified the S. pombe gene encoding adenylyl cyclase (Young et al., Proc. Natl. Acad. Sci. 86: 7989 [1989]). This gene encodes a protein that is highly similar to S. cerevisiae CYR1, including the carboxy-terminal catalytic domain and the leucine-rich repeats. Amino-terminal to the repeats is complete divergence. Overexpression of S. pombe CYR1 leads to sterility in that organism. We have begun to look for suppressors of this phenotype. It is our expectation that S. pombe contains another RAS-like protein that controls its adenylyl cyclase activity.

The availability of the S. pombe system will enable us to examine the functions of S. cerevisiae genes more clearly. For example, we have already found that antibodies to S. cerevisiae CAP can be used to precipitate S. pombe adenylyl cyclase activity. A homolog to CAP must exist in this organism, and
The parallels between the mammalian and yeast RAS pathways are striking. First, mammalian RAS can function in a yeast host and can stimulate yeast adenylyl cyclase in vivo and in vitro. Second, yeast RAS can function in mammalian cells to induce transformation (DeFeo-Jones et al., Science 228: 179 [1985]). Third, mammalian GAP can complement yeast deficient in RAP1. Fourth, we and other investigators have shown that Ha-ras mutants that are dominant interfering in yeast are dominant interfering in mammalian cells (Powers et al., Cell 36: 607 [1984]; Feig and Cooper, Mol. Cell. Biol. 8: 3235 [1988]). Most recently, we have shown that the yeast SCD25 gene, a homolog of CDC25 (Boy-Marcotte et al., Gene 77: 21 [1989]), can transform animal cells. Despite these similarities, the targets of RAS action appear to be different in yeast and vertebrates.

We have applied a genetic approach to identifying mammalian components that interact with RAS. We constructed mammalian cDNA libraries in yeast expression vectors and have searched these libraries for genes that can suppress mutations in the RAS pathway in yeast (Colicelli et al., Proc. Natl. Acad. Sci. 86: 3599 [1989]). We have utilized three types of screens, using both S. cerevisiae and S. pombe as hosts.

The first cDNA screen has been for genes that can suppress the heat-shock phenotype of cells with the RAS2V19 mutation. The rationale for this screen is that, like the defective adenyl cyclase gene, the mammalian effector of RAS might well interfere with activated RAS. Several candidates have emerged from this screen. One set encodes cAMP phosphodiesterases (see below). The second set is under evaluation. Genes from the second set do not appear to encode cAMP phosphodiesterases and bear no homology with previously identified nucleotide sequences in the data banks. At least one member of this set encodes a product that interferes with activated ras1 in S. pombe. We expect that genes from this set either encode true inhibitors of RAS function (e.g., by blocking nucleotide exchange) or, more likely, are effectors of RAS that are not functional in yeast hosts.

In the second cDNA screen, we have sought cDNA genes that can suppress the loss of ras1 in S. pombe. Several mammalian cDNAs have been isolated this way, and they are still under investigation. Among the genes so isolated have been Ha-ras, RAP1, and RAP1A. The latter two genes are members of the RAS superfamily (Pizon et al., Oncogene 3: 201 [1988]). RAP1 was also isolated as Ki-rev-1, a gene capable of suppressing Ki-ras function in mammalian cells (Kitayama et al., Cell 56: 77 [1989]). Our results demonstrate that RAP genes and RAS genes can share effectors.

The third cDNA screen is designed to search for genes that can suppress S. cerevisiae with temperature-sensitive alleles of CDC25. We hope that this screen uncovers mammalian homologs of CDC25.
ameyer and Wang, *J. Virol.* 53: 879 [1985]). We first encountered it as MCF3, an oncogene arising by rearrangement following a DNA transfer experiment. Subsequent studies revealed ROS to be expressed in a high proportion of human glioblastomas, but in very few other kinds of tumors, and not at all in normal glial cells. In glioblastomas, the predominant mRNA is about 8.0 kb. We have cloned cDNA to this mRNA from SW1088 and have completed the nucleotide sequence (Birchmeier et al., *Cell* 43: 615 [1985]). We have also cloned a cDNA to a 4.0-kb ROS mRNA found in another cell line, U118 (Sharma et al., *Oncogene Res.* 5: 91 [1989]). ROS can encode a transmembrane tyrosine kinase that shares homology with the product of the *Drosophila melanogaster* sevenless gene, both in the intracellular kinase domain and in the extracellular domain. Studies of ROS have shown that in two out of two glioblastomas, it has undergone mutation, consistent with the idea that the aberrant expression of ROS contributes to the malignancy of tumors of glial origins. We plan to continue these studies, examining the expression of ROS in primary brain tumors, using antisera to a ROS protein that we have developed. It is our hope that ROS protein will prove to be a useful cell surface marker for the diagnosis and treatment of tumors of glial origin.

A New Method for Genomic Difference Cloning

I Wieland, G. Bolger, K. O'Neill, G. Asouline

One common and fundamental problem of molecular biology confronts us when two similar genomes differ and we desire to understand the difference. One simple form of this problem can occur when a genome becomes deleted for sequences present in another due to germ line mutation, as can happen in genetic disease, or due to somatic mutation, as can happen during the development of cancer. Differences can also be acquired by infection with a DNA-based pathogen. Methods for identifying and isolating sequences present in one DNA population that are absent or reduced in another are called “difference cloning.” Methods for difference cloning of cDNA populations have been widely described. Only one method for the difference cloning of genomic DNA is reported in the literature. This method was first described by Lamar and Palmer (*Cell* 37: 171 [1984]), who used it to clone sequences from the Y chromosome. Kunkel and co-workers used a variation of this method to clone fragments of the Duchenne’s muscular dystrophy locus (Kunkel et al., *Proc. Natl. Acad. Sci.* 82: 4778 [1985]), which becomes deleted in some afflicted individuals. We have developed a different method for genomic difference cloning that is potentially more powerful (Wieland et al., *Proc. Natl. Acad. Sci.* [1990] in press). Although our method is not yet sufficient to isolate and define the small differences in genomes that would make it enormously useful as a tool for the study of neoplasia or infectious disease of viral origin, improvements are under way that will bring our method into that range. In its present state, our method is useful for the analysis of some genetic diseases and infectious diseases of unknown origin.

In our procedure for genomic difference cloning, sequences present in one genomic DNA population (“tester”) are isolated that are absent in another (“driver”). By subtractive hybridization, a large excess of driver is used to remove sequences common to a biotinylated tester, enriching the “target” sequences that are unique to the tester. After repeated subtractive hybridization cycles, tester is separated from driver by avidin/biotin affinity chromatogra-
phy, and single-stranded target is amplified by the polymerase chain reaction, rendering it double-stranded and clonable. We have successfully modeled two situations: the gain of sequences that result from infection with a pathogen and the loss of sequences that result from a large hemizygous deletion. We obtain 100–700-fold enrichment of target sequences. We are in the process of improving the method by exploiting the second-order kinetics of self annealing. We are hopeful that we can soon begin to identify very small sequence differences between chromosomes.

**PUBLICATIONS**


**In Press, Submitted, and In Preparation**


